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Note

Direct determination of dextromethorphan and its three metabolites in urine by high-performance liquid chromatography using a precolumn switching system for sample clean-up

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Dextromethorphan (I) is an antitussive drug, which is mainly metabolized via oxidative O-demethylation to yield dextrorphan (II). Minor N- and N,O-demethylation products, 3-methoxymorphinan (III) and morphinan-3-ol (IV), respectively, are also produced. Important inter-individual differences in dextromethorphan O-demethylation have been reported [1], and a study of the co-segregation of dextromethorphan O-demethylation with debrisoquine 4-hydroxylation demonstrated a complete concordance of the two phenotypic assignments [2,3]. I thus appears to be an interesting substance for human pharmacogenetic investigations. These are based on the determination of the I/II metabolic ratio. Chromatographic methods used to determine I and its metabo-

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lites include gas chromatography (GC) [4,5] and high-performance liquid chromatography (HPLC) [6–8]. Among the HPLC techniques, the one proposed by Achari et al. [7] only measures I. The technique of Park et al. [6] concerns I and its metabolites, but the extraction step is tedious and time-consuming. The problem is the same for the method published by East and Dye [8]. Moreover, for this last method, I and II are extracted and analysed separately.

The aim of the present study was therefore to develop an accurate, sensitive and fast method for the simultaneous measurement of I and its metabolites in urine in order to aid pharmacogenetic studies.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents used were of analytical-reagent grade. I and II were kindly supplied by Hoffman-LaRoche (Basle, Switzerland). III and IV were synthesized in our laboratory (Organic Chemistry Service) by a previously described method [9]. Human urine samples free of any drugs were obtained from healthy volunteers.

Enzymatic hydrolysis

To 1 ml of urine were added 1 ml of 0.1 *M* acetate buffer (pH 5), 20 μl of β-glucuronidase/β-arylsulphatase from *Helix pomatia* (Boehringer Mannheim) and 50 μl of 0.6 *M* aqueous sodium azide. The sample was then incubated at 37 °C for 12 h, a sufficient period for complete cleavage of the conjugates.

Analytical procedure

The analytical system (Fig. 1) consisted of two solvent-delivery pumps (Waters 6000A, Waters Assoc., Milford, MA, U.S.A. and Merck-Hitachi 655A, E. Merck, Darmstadt, F.R.G.) and a Rheodyne 7000 six-way valve (Rheodyne, Cotati, CA, U.S.A.). A UV–visible detector operating at 200 nm, 0.02 a.u.f.s. (Merck-LMC system SM25) was used.

Hydrolysed urine samples (100 μl) were directly injected on a precolumn dry-packed with LiChrosorb CN (Hibar RT 30×4 mm I.D.; 10 μm particle size, E.

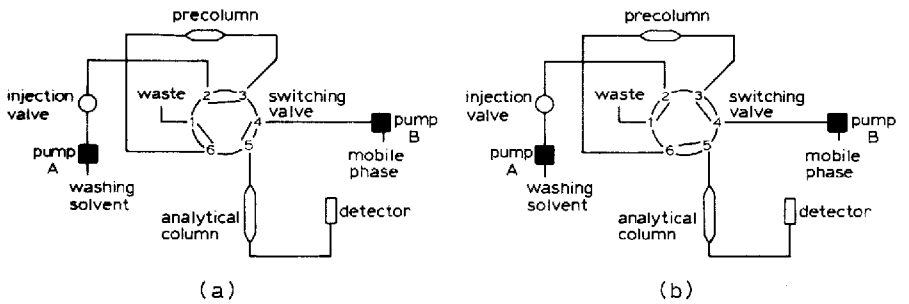

Fig. 1. Schematic representation of the column-switching system used for the analysis of dextromethorphan and its metabolites in urine. (a) Sample clean-up, (b) sample elution.

Merck) and flushed at a flow-rate of 0.5 ml/min with $10^{-2}M$ potassium dihydrogenphosphate–acetonitrile (50:50, v/v) and adjusted to pH 4 with phosphoric acid (sample clean-up step). Exactly 3 min after injection, the valve was switched over and the sample was eluted onto the analytical column (Spherisorb phenyl 250×4.6 mm I.D., 5 μm particle size, Société Française Chromato colonne) with the same mobile phase at a flow-rate of 1.4 ml/min (pressure 14 MPa) (sample elution step). The system was operated at room temperature.

Preparation of calibration curves

Aliquots of I in methanol varying from 0.3 to 8.4 μg and of II in methanol varying from 1.2 to 43.7 μg were introduced in conical tubes. Methanol was then evaporated under a nitrogen stream, and 1 ml each of blank urine and the hydrolysis medium were added. After incubation, 100 μl were analysed as previously described.

RESULTS AND DISCUSSION

I has two absorption maxima at 200 and 280 nm. The initial experiments were carried out at 280 nm, but owing to a lack of sensitivity at this wavelength, 200 nm was finally selected. Under these conditions, the detection limit was less than 0.04 μg/ml for I and 0.05 μg/ml for II (signal-to-noise ratio of 3 and 4, respectively). These values are greater than those reported by East and Dye [8] and Achari et al. [7] (0.02 μg/ml for I), but lower than those reported by Park et al. [6] (0.11 μg/ml for I).

Moreover, two important points have to be considered. These values are sufficient for pharmacogenetic studies. They permit unambiguous distinction between poor and extensive metabolizers, which is the aim of the proposed method. If desired, the sensitivity could be increased by using a fluorescence detector (excitation at 280 nm, emission at 310 nm).

Fig. 2 shows typical chromatograms. No endogenous sources of interferences were observed (Fig. 2A). Fig. 2B shows a chromatogram of urine spiked with I and its three metabolites, and Fig. 2C is a chromatogram of a volunteer who was classified as poor metabolizer. In all cases, I and its metabolites are well resolved.

Standard curves were constructed by plotting peak areas versus concentrations of drug and the demethylated metabolite II. Instrument response and concentrations were linearly related for both I and II over ranges of 0.5–7 and 1–40 μg/ml, respectively. The linear regression curves are described by the equations $y=11472x-1273$ ($r=0.997$) for I and $y=9067x-2656$ ($r=0.996$) for II (x=concentration of molecules in μg/ml of urine, y=peak area in μV). No calibration curves were constructed for III and IV because only the I/II metabolic ratio is used for pharmacogenetic studies.

The precision of the method was evaluated at two concentrations of I (1.76 and 3.69 μg/ml) and II (9 and 27 μg/ml) by analysing each one six times on the same day. The data in Table I demonstrate the high degree of precision of the method.

Table II shows that the coefficient of variation (C.V.) of the method did not

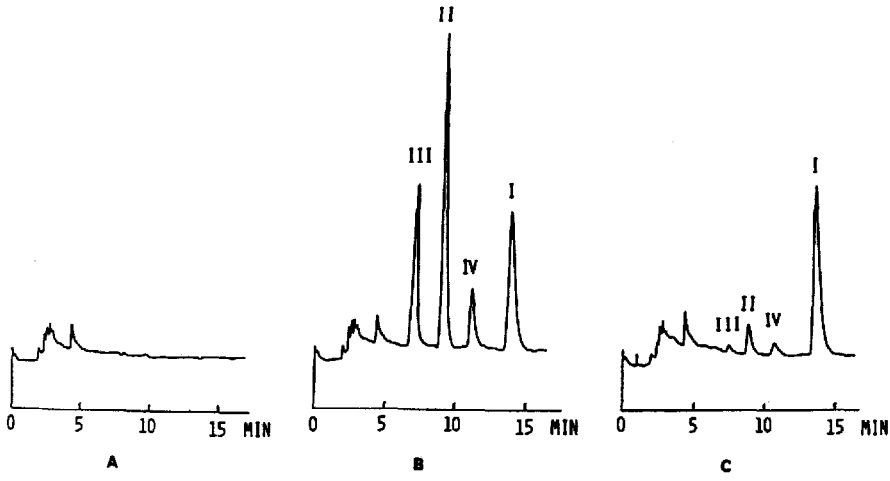

Fig. 2. Chromatographic profiles obtained with HPLC column switching of drug-free urine (A), urine spiked with 1.4 μg/ml dextromethorphan (I), 5.14 μg/ml dextrorphan (II), 3-methoxymorphinan (III) and morphinan-3-ol (IV) (B), and urine from a poor metabolizer receiving 30 mg of dextromethorphan (C) (dextromethorphan: 1.709 μg/ml, dextrorphan: 0.585 μg/ml). The elution times are given as a function of the time of column-switching.

TABLE I

PRECISION AND ACCURACY OF THE HPLC ASSAY FOR DEXTROMETHORPHAN AND DEXTRORPHAN IN URINE

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D., $n=6$) (μg/ml)	Coefficient of variation (%)
Dextromethorphan	1.76	1.70 ± 0.07	4.48
	3.69	3.64 ± 0.09	2.7
Dextrorphan	9	8.92 ± 0.23	2.63
	27	26.96 ± 0.25	0.78

TABLE II

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION FOR DEXTROMETHORPHAN AND DEXTRORPHAN

Compound	Assay	Concentration (μg/ml)	Coefficient of variation (%)
Dextromethorphan	Intra-assay	0.704	2.51
	($n=6$)	4.928	1.64
	Inter-assay	0.704	3.88
	($n=6$)	4.928	3.40
Dextrorphan	Intra-assay	2.570	3.06
	($n=6$)	30.880	2.71
	Inter-assay	2.570	4.20
	($n=6$)	30.880	3.80

TABLE III

URINARY LEVELS OF I AND II, AND THE I/II RATIO FOR EIGHT NORMAL SUBJECTS

Subject	Concentration (μg/ml)		I/II ratio
	I	II	
1	0.100	76.06	0.0013
2	0.467	68.47	0.0068
3	0.140	19.30	0.0072
4	0.771	37.58	0.0205
5	0.469	20.93	0.0240
6	1.350	47.21	0.0285
7	0.950	2.06	0.4611
8	1.709	0.58	2.9465

exceed 4.2% for replicate analysis of I and II in intra- and inter-assay variation studies.

The proposed switching technique has been applied to the determination of the metabolic status of eight volunteers (Table III). Six of them turned out to be extensive metabolizers ($0.0013 < I/II < 0.4611$) and two poor metabolizers ($I/II > 0.4611$).

CONCLUSION

In drug measurements, the tedious step is often the extraction from biological fluids. This is particularly true in pharmacogenetic studies where a great number of samples have to be analysed. Previous methods for the measurement of I and II are time-consuming: ca. 100 and 60 min for the techniques of Park et al. [6] and East and Dye [8], respectively. With the method that we propose, this time is reduced to less than 20 min. The choice of precolumn and column types, the mobile phase composition, the flow-rates and the wash-time were optimized to avoid contamination of the analytical column and to minimize the analysis time. More than 60 samples can be injected before the precolumn has to be changed as a result of increasing pressure in the analytical system. Although they are not needed in pharmacogenetic studies, III and IV may also be measured with the proposed method.

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